IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

		Atty. Docket: KARAOLIS1A
In re Application of:	)	Conf. No.: 2282
David K. R. KARAOLIS	)	Art Unit: 1645
Appln. No.: 10/565,591	)	Examiner: N. Archie
Filed: October 6, 2006	)	Washington, D.C.
For: METHOD FOR ATTENTUATING…	)	

DECLARATION UNDER 37 CFR §1.132

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop
401 Dulany Street
Alexandria, VA 22314

Sir:

I David K. R. KARAOLIS, hereby declare and state as follows:

I am the same David K. R. Karaolis listed in the above-identified application as the sole inventor and my educational and professional experience is presented in the curriculum vitae attached hereto.

In the first enablement rejection, I understand that the examiner has taken the position that the claimed invention is not enabled for any method of attenuating the virulence of any microbial pathogen or for inhibiting or reducing colonization by any microbial pathogen in a patient thereof,

comprising administering to a patient in need an effective amount of c-di-GMP or a cyclic dinucleotide. In the second enablement rejection, I understand that the examiner has also taken the position that the claimed invention of claims 17-21 is not enabled for any method for inhibiting bacterial colonization and biofilm formation or for reducing colonization and pre-formed bacterial biofilm on a solid surface, comprising exposing the solid surface to an effective amount of c-di-GMP or a cyclic dinucleotide.

Attached hereto as Exhibit 1 are copies of two abstracts (Kumagai et al. and Petersen et al.) presented at the 2009 American Society for Microbiology general meeting in Philadelphia, PA. Also attached hereto as Exhibit 2 is a copy of Mano et al., "Synthesis of Cyclic Bis(3'-5')-2'-deoxyguanylic/guanylic Acid (c-dGpGp) and Its Biological Activities to Microbes", *ChemMedChem* 2:1410-1413 (2007). The contents of both exhibits are discussed below as they relate to the enablement rejections.

I will address the enablement rejections below as they relate to bacterial pathogens rather than the entire scope of microbial pathogens.

With regard to the first enablement rejection, the examiner states that the specification does not give any working example (i.e., challenged mice models or passive

immunization approaches). This statement is clearly incorrect. Example 8 on page 80 of the specification teaches that "Treatment with c-di-GMP clearly show a significant dose-dependent suppressing effect (reduction in CFU counts) of c-di-GMP on the ability of *S. aureus* to multiply or colonize in the mammary gland (Fig. 15). The results show that 50 nanomoles of c-di-GMP injected into the mammary gland *in vivo* significantly inhibits *S. aureus* infection of the mammary gland by at least 10-fold (T test: p=0.004; Mann-Whitney U-test: p=0.009)."

Moreover, the abstract in Exhibit 1 from Petersen et al. presents data on the role of c-di-GMP in virulence of *Brucella melitensis* (gram-negative intracellular bacterial pathogen). Several deletion mutants were studied for their virulence *in vivo* in mice and it was found that genes involved in regulation of c-di-GMP had altered virulence. The results demonstrate a role for c-di-GMP in virulence in which high levels of c-di-GMP (similar to what is expected if c-di-GMP is added exogenously) decrease virulence while low levels increase virulence. One of skill in the art would certainly be enabled for administration of c-di-GMP *in vivo* to reduce the virulence of *Brucella*.

I have previously disclosed in the present specification at paragraph [0044]-[0046] that c-di-GMP may act

to inhibit biofilm formation/colonization/virulence in some bacteria or it may act in the opposite manner and induce or enhance biofilm formation/colonization/virulence in others. Thus, specific cyclic dinucleotides may act as either agonists or antagonists of c-di-GMP, a property that can be rapidly and readily determined with only routine experimentation using biofilm formation/inhibition assays in microtiter plates, test tubes or flasks, as disclosed in paragraph [0045] and in the examples of the specification.

The abstract in Exhibit 1 from Kumagai et al. presents data that c-di-GMP regulates bacterial internalization of *Ehrlichia chaffeensis*, an intracellular gram negative bacterial pathogen similar to *Rickettsia*, into host cells and intracellular growth. To test the hypothesis that c-di-GMP plays a role in intracellular infection, the function of c-di-GMP was studied using a c-di-GMP analogue (TBDMS-c-di-GMP, or CDGA, which is a cyclic dinucleotide). When *E. chaffeensis* bacterial cells were exposed to TBDMS-c-di-GMP, some outer membrane proteins were down regulated and internalization into host cells was impaired. When TBDMS-c-di-GMP was added to infected cells at exponential growth stage of *E. chaffeensis*, bacterial proliferation was inihibited and the *E. chaffeensis* inclusion was malformed. The data thus show to one of skill in the art that TBDMS-c-di-GMP can

function as a c-di-GMP antagonist, and that c-di-GMP regulates bacterial outer membrane protein expression, thereby being involved in signal transduction across cell membranes for bacterial internalization and intracellular growth. The results suggest to one of skill in the art that treatment with c-di-GMP or a cyclic dinucleotide analogue can inhibit virulence.

The two abstracts discussed above and my 1.132 declaration submitted with the amendment filed October 8, 2008, which presents experimental results demonstrating that c-di-GMP significantly inhibits microbial colonization, virulence and infection against intranasal (i.n.) or intraperitoneal (i.p.) challenge with various microbial pathogens, including gram positive and gram negative bacteria, show that the inhibition of microbial colonization and reduction of virulence with cyclic dinucleotides span a wide range of species within the genus of bacterial pathogens. Accordingly, one of ordinary skill in the art would readily believe and expect that the presently claimed methods would be applicable to the entire genus of bacterial pathogens.

With regard to the second enablement rejection, the attached Mano et al. article presents results demonstrating that two cyclic dinucleotides, c-di-GMP and c-dGpGp, inhibited biofilm formation of three different types of bacteria,

Pseudomonas aeruginosa (gram positive), *Vibrio parahaemolyticus* (gram negative), and *Staphylococcus aureus* (gram positive), on a polystyrene solid surface (see page 1410, second full paragraph in right column). These results, while not conducted in my laboratory, nevertheless demonstrate that the presently claimed method for inhibiting bacterial colonization and biofilm formation on a solid surface (polystyrene bead) is indeed enabled.

In conclusion, the presently claimed methods are indeed fully enabling to one of skill in the art for the full scope of the recited bacterial pathogens and cyclic dinucleotides.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

14 September 2009
Date

/David K.R. Karaolis/
David K. R. KARAOLIS

October 2007

CURRICULUM VITAE

DAVID K. R. KARAOLIS, Ph.D.

Bacteriology Manager/Director
National Biodefense Analysis and Countermeasures Center (NBACC)
Frederick, MD 21702
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CITIZENSHIP United States

Security Clearance: SECRET- Current
DOJ Select Agent Clearance: Current
CDC Import and Transfer Permit: Etiologic Agents or Vectors of Human Disease- Current
USDA Import and Transport Permit: Controlled Materials and Organisms and Vectors- Current
Immunizations: Anthrax, Tularemia, BOT, Hep B, Tetanus

EDUCATION

1985	Matriculation	Newington College, Sydney, Australia
1986-1990	B. Sc.	Department of Life Sciences The University of Technology, Sydney, Australia
1990-1991	Honors	Department of Life Sciences The University of Technology, Sydney, Australia
1991-1994	Ph.D.	Department of Microbiology, The University of Sydney, Australia
1995-1998	Postdoctoral	Center for Vaccine Development, Department of Medicine University of Maryland School of Medicine/ VA Medical Center, Baltimore

SCIENTIFIC AND MANAGEMENT EXPERIENCE

Ph.D. Microbiologist (security clearance) with 20 years experience in a broad range of applied scientific fields including of clinical microbiology, microbial bioforensics, virulence assessment and characterization, molecular pathogenesis, antibiotics, vaccine and drug development. Experienced in laboratory procedures in the culture, identification, characterization and manipulation of both BSL-2 and BSL-3 select agents, including biothreat agents important in biodefense, as well as studying virulence assessment and host response using in vitro and in vivo animal models. Five (5) patents involving infectious disease, host response and therapeutics.

As the Director of Bacteriology at the National Biodefense Analysis and Countermeasures Center (NBACC), comprising both the National Bioforensic Analysis Center (NBFAC) and National Biothreat Characterization Center (NBTCC), I manage and direct bacteriology capabilities including bacterial diagnostics, applied research studies and the development of new technologies. My tenure has included the establishment of the NBACC/NBFAC bacteriology BSL-3 containment laboratory, as well as successfully obtaining ISO 17025 accreditation of bioforensic bacteriology operations under the ISO Quality Management System (QMS). Extensive

management experience in the strategic planning, coordination and program management of multidisciplinary national and international projects/programs and liason with government/private organizations, including the design and implementation of project goals, allocation of budget/resources, data analysis and reporting.

In addition, I have also pioneered the discovery and development of a novel drug-platform technology for commercialization. This work involves immunomodulator molecules and includes several patentable technologies and clinical applications including new immunoprophylactic, immunotherapeutic and vaccine approaches for preventing and treating infectious diseases and cancer.

AWARDS

1998-2003	Burroughs Wellcome Fund Career Award in the Biomedical Sciences
2005-2006	Burroughs Wellcome Fund Career Award (Supplemental award)
2006	Department of Homeland Security- Certificate of Recognition

EMPLOYMENT

2006-present	Bacteriology Manager/Director National Bioforensic Analysis Center (NBFAC) National Biodefense Analysis and Countermeasures Center (NBACC)
2006	Assistant Professor (adjunct) Department of Pediatrics University of Maryland School of Medicine
1999-2006	Assistant Professor Department of Epidemiology and Preventive Medicine University of Maryland School of Medicine
1999-present	Faculty Member (affiliate) Molecular and Cell Biology Graduate Program University of Maryland School of Medicine
1999-present	Assistant Professor (affiliate) Department of Medicine University of Maryland School of Medicine
1998-1999	Instructor of Medicine Division of Hospital Epidemiology University of Maryland School of Medicine
1995-1998	Postdoctoral Fellow Center for Vaccine Development University of Maryland School of Medicine
1991-1995	Clinical Microbiologist Department of Microbiology Hanly Moir Private Pathology Laboratories, Sydney
1991-1994	Research Assistant Department of Microbiology

The University of Sydney

1991-1994 Tutor/Teacher
Department of Life Sciences
The University of Sydney

1990-1991 Clinical Microbiologist
Department of Microbiology
The Royal North Shore Hospital, Sydney

1990-1991 Laboratory Demonstrator
Department of Microbiology
University of Technology, Sydney

1987-1989 Trainee Microbiologist
Department of Microbiology
Royal North Shore Hospital, Sydney

PROJECT MANAGEMENT COURSES

2002 Burroughs Wellcome Fund and Howard Hughes Medical Institute Course in Scientific Management

PROFESSIONAL ASSOCIATIONS

1988-present American Society for Microbiology
1988-present Australian Society for Microbiology
1998-present American Academy for the Advancement of Science

EDITORIAL TASKS

1996-present Ad Hoc Reviewer, Royal Society of Tropical Medicine and Hygiene
1999-present Ad Hoc Reviewer, Trends in Microbiology
1999-present Ad Hoc Reviewer, Infection and Immunity
2001-present Ad Hoc Reviewer, Journal of Antimicrobial Chemotherapy
2002-present Ad Hoc Reviewer, Microbiology
2002-present Ad Hoc Reviewer, Journal of Clinical Microbiology
2002-present Ad Hoc Reviewer, Journal of Infectious Diseases
2003-present Ad Hoc Reviewer, Molecular Microbiology

GRANT REVIEW WORK

1995-1998 USAID Office of Health and Nutrition
2003 The Wellcome Trust (United Kingdom)
2004 Science Foundation Ireland (SFI)
2005 U.S. Department of the Army

PATENTS

- Bacteriophage-based vaccines and detection systems, methods of using same, and products thereof.
Karaolis, D.K.R. U.S. Serial # 60/133373
- Method and system for direct detection of fungal pathogens.

Karaolis, D.K.R. U.S. Serial # 60/545,895
- Method for attenuating virulence of microbial pathogens and for inhibiting microbial biofilm formation.

Karaolis, D.K.R. PCT/US04/23498
- Method for stimulating the immune, inflammatory or neuroprotective response.

Karaolis, D.K.R. U.S. 11/079,886; PCT/US05/08447
- A method for inhibiting cancer cell proliferation or increasing cancer cell apoptosis.

Karaolis, D.K.R. U.S. 11/079,779; PCT/US05/08448

UNIVERSITY OF MARYLAND COMMITTEES AND ACTIVITIES

University of Maryland Committees:

2000–present	UMD Recombinant DNA Committee
2000–present	UMD Institutional Bio-Safety Committee

School of Medicine Committees:

1999-2000	Scientific Review Committee on NIH Program Project, Molecular and Cellular Pathogenesis of Urinary Tract Infection, J. Warren , Principle Investigator.
1999-2000	Scientific advisory committee for Health Sciences Facility II
1999	Judge for Graduate Research Conference Day
2000-2003	Alt. Representative for Faculty Council

Departmental Committees:

1999-2004	Research Committee, DEPM
1999-2002	Seminar Committee, DEPM
2001-2002	Graduate Admissions Committee, DEPM
2002-2003	Resource Allocation for Teaching and Service Committee, DEPM

VETERANS AFFAIRS COMMITTEES:

2002-present	Biosafety Committee, VA Medical Center, Baltimore

TEACHING ACTIVITIES

Teaching at University of Maryland School of Medicine:

1999-present	Bacterial Genetics MMIC/DMIC 635 (Graduate Students)

Teaching at other universities:

1990-1991	Clinical Microbiology Department of Microbiology University of Technology, Sydney
1991-1994	Microbiology

Department of Life Sciences
The University of Sydney

MENTORSHIP at UMB

Instructors (Faculty):

Afsar Ali, Ph. D. (2000-2003)

Postdoctoral fellows:

Jing Wang, M.D., Ph. D. (1999-9/2001)
Dalin Zhang, Ph.D. (1999-2003)
Afsar Ali, Ph. D. (2000)
Rajanna Chythanya (2001-2005)

Graduate students:

Mohammed Harun Rashid (2000-present)

Ph.D. Rotation

Amanda King (2000) – MCB Program
Jessina McGregor (2002) – DEPM Program
Simone Shurland (2003) DEPM program

Ph.D. Committee Member

Christopher J. Grim (Advisor: Rita R. Colwell)

UMD Research Training Program

Layla Lavasani (2002) – NIEHS (Minority) Toxicology Program, UMB
Tamara Webster (2003) - NIEHS (Minority) Toxicology Program, UMB
Keisha Findley (2003-2004) – MARC (Minority) Program, UMBC
Tara Brinck (2004) – Fogarty Minority International Training Program, UMBC

INVITED TALKS

1998 Karolinska Institute, Stockholm, Sweden. Analysis of the enteropathogenic *E. coli* LEE pathogenicity island: RDEC as a model.

1998 University of Sydney, Dept. of Microbiology. Genetic analysis of the *Vibrio* pathogenicity island.

1999 99th General Meeting of the American Society for Microbiology, Chicago, IL. Session: Phage and virulence; A bacteriophage encoding a pathogenicity island and type IV pilus in *V. cholerae*.

1999 16th Biennial conference on Virus and Phage Assembly. Rio Rico, AZ. A bacteriophage encoding a pathogenicity island and type IV pilus in *Vibrio cholerae*.

1999 XX SBM Congress, Brazilian Society for Microbiology, Salvador, Brazil. Genetics of virulence and evolution of *Vibrio cholerae*.

1999 XX SBM Congress, Brazilian Society for Microbiology, Salvador, Brazil. The *Vibrio cholerae* pathogenicity island.

1999 48th Annual Meeting of the American Society for Tropical Medicine and Hygiene, Washington, DC. Cholera and phage: genetic rearrangements, the *Vibrio* pathogenicity island, and prospects for emergence of new pandemic strains.

1999 FDA, Bethesda, Maryland. Epidemic cholera and phage: Role of phage in epidemic cholera.

2000 19th Annual Meeting of the American Society for Virology, Fort Collins, CO. Session: Viral virulence, pathogenesis and immunity; Bacteriophage-encoded virulence factors in *V. cholerae*.

2000 100th General Meeting of the American Society for Microbiology, Los Angeles, CA. Session: Interacting DNA elements, pathogenesis, and bacterial apoptosis; Virulence-conferring phage in *Vibrio cholerae*.

2002 Southwestern branch of the American Society for Microbiology, Annual Meeting, Gainsville, Fl. Session: Food Microbiology: Epidemic *V. cholerae*: PAIs, polysaccharide and persistence

2002 Thomas Jefferson University, Dept. of Biochemistry. Epidemic *V. cholerae*: Pathogenicity islands, polysaccharides and persistence.

2003 University of Sydney, School of Molecular Biosciences. Epidemic *V. cholerae*: Pathogenicity islands, polysaccharides and persistence.

2003 University of New South Wales, School of Biotechnology and Biomolecular Sciences. Epidemic Cholera: Importance of Pathogenicity islands and Exopolysaccharides.

2004 Johns Hopkins Hospital Bloomberg School of Public Health. *Vibrio cholerae* molecular pathogenesis.

2004 Catholic University of America, Department of Biology. *Vibrio cholerae* pathogenesis: new molecular insights and identification of a novel class of signaling (therapeutic?) molecule.

2004 Nabi Biopharmaceuticals. Cyclic Dinucleotides: a Novel Drug-Platform.

2005 Schering Plough. Cyclic Dinucleotides: a Novel Drug-Platform

2006 1st World Congress: Alliance for the Prudent use of Antibiotics (APUA). Antibiotic resistance in bioterror threats. Boston, MA. December 11-12.

ABSTRACTS

1. **Karaolis, D.K.R.**, R. Lan, PR. Reeves. 1994.Annual Meeting of the Australian Society for Microbiology, Melbourne, Victoria, Australia. Molecular evolution of the 7th pandemic clone of *Vibrio cholerae* and its relationship to other pandemic and epidemic strains. (Oral).

2. **Karaolis, D.K.R.**, R. Lan, PR. Reeves. 1995. 95th General Meeting of the American Society for Microbiology. Washington, DC. The 6th and 7th cholera pandemics are independent clones derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*.

3. **Karaolis, D.K.R.**, T.K. McDaniel, and E.C. Boedeker. 1995. Cloning of the RDEC-1 locus of enterocyte effacement (LEE) and functional analysis of its phenotype on Hep-2 cells. *Advances in Experimental Medicine and Biology*. Proceedings of the First International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases, Mt. Rushmore, SD. Plenum Press. p241-245.

4. **Karaolis, D.K.R.**, T.K. McDaniel, J.B. Kaper, and E.C. Boedeker. 1996. Cloning of the RDEC-1 locus of enterocyte effacement (LEE) and functional analysis of the phenotype on HEp-2 cells. 96th General Meeting of the American Society for Microbiology. New Orleans, LA. Abstract B-90.

5. **Karaolis, D.K.R.**, R.Lan, P.R. Reeves. 1997. The *aldA* gene of *Vibrio cholerae* is a genetic marker for strains with pandemic potential. From the Proceedings of the 31st U.S.-Japan Joint Conference on Cholera and Related Diarrheal Disease, Kiawah Island, South Carolina, USA. 1995. *In*: Cytokines, Cholera, and the gut. G.T. Keusch and M. Kawakami *Eds*. IOS Press.p213-217.

6. **Karaolis, D.K.R.**, S. Sozhamannan, J.A. Johnson, J.B. Kaper. 1998. 98th General Meeting of the American Society for Microbiology. Atlanta, GA. Novel non-O1/non-O139 *Vibrio cholerae* containing the VPI and CTX. Abstract B-179.

7. Lipp, E.K., I.N.G. Rivera, M. Talledo, A. Neale, **D.K.R. Karaolis**, A. Huq, R.R. Colwell. 2001. 101st General Meeting of the American Society for Microbiology. Orlando, FL. Optimal conditions for infection and multiplication of *Vibrio cholerae* specific phages isolated from seawater.

8. Vital-Brazil, J.M., **D.K.R. Karaolis**, D.P. Rodrigues, L.C. Campos. 2001. 101st General Meeting of the American Society for Microbiology. Orlando, FL. Prevalence of virulence-associated genes in clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991-1999.

9. Wang, J. J. Xu, A. Ali, **D.K.R.Karaolis**. 2001. 101st General Meeting of the American Society for Microbiology. Orlando, FL. Genetic analysis of the plasmid form of the *Vibrio cholerae* pathogeneicity island.

10. Zhang, D. S. Rao, **D.K.R. Karaolis**. 2001. 101st General Meeting of the American Society for Microbiology. Orlando, FL. Functional analysis of Orf4 encoded by the *Vibrio cholerae* pathogenicity island.

11. Zhang, D., W. Sun, Z. Xu, **D.K.R. Karaolis**. 2002. 102st General Meeting of the American Society for Microbiology. Salt Lake City, UT. The VPI-encoded Orf4 modulates secreted proteins in *Vibrio cholerae*.

12. Rashid, M. H., A. Ali, **D.K.R. Karaolis**. 2002. 102st General Meeting of the American Society for Microbiology. Salt Lake City, UT. Analysis of the Genetic Switch for Phenotypic Conversion Between the Smooth and Rugose Exopolysaccharide Phenotypes of *V. cholerae.*

13. Rashid, M. H., A. Ali, **D.K.R. Karaolis**. 2003. 103st General Meeting of the American Society for Microbiology. Washington, D.C. Genetic analysis of high frequency rugose exopolysaccharide production (HFRP) in epidemic *V. cholerae.*

14. Rajanna, C. and **D.K.R. Karaolis**. 2003. 103st General Meeting of the American Society for Microbiology. Washington, D.C. The VPI-encoded Int and VpiT of epidemic *V. cholerae* have roles in high frequency rugose exopolysaccharide production (HFRP).

15. Zhang, D., Sun, W. and **D. K. R. Karaolis**. 2003. 103st General Meeting of the American Society for Microbiology. Washington, D.C. The *Vibrio* pathogenicity island *mop* modulates cholera toxin, motility and biofilm formation in epidemic *V. cholerae.*

16. Rajanna, C., Rashid, M.H. and **D.K.R. Karaolis**. 2004. 104th General Meeting of the American Society for Microbiology. New Orleans. Regulation of *Vibrio cholerae* biofilm formation and intestinal colonization by *Vibrio* pathogenicity island recombinases.

17. Zhang, D., Rajanna, C. and **D.K.R. Karaolis**. 2004. 104th General Meeting of the American Society for Microbiology. New Orleans. Recombinase-mediated control of cholera toxin in epidemic *Vibrio cholerae.*

18. **Karaolis, D.K.R.**, Rashid, M.H. Rajanna, C., Buckles, E., Luo, W. and Hayakawa, Y. 2004. 44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Washington, D.C. c-di-GMP as a novel anti-biofilm agent against *Staphylococcus aureus.*

19. **Karaolis, D.K.R.**, Means T.K., Brouillette, E., Talbot, B.G., Yang, D., Muraille, E., Hyodo, M., Hayakawa, Y. and Malouin, F. 2006. General meeting of the Amercan Society for Microbiology. Orlando. c-di-GMP is an immunostimulatory molecule with prophylactic and adjuvant activity.

PUBLICATIONS

BOOK CHAPTERS

1. **Karaolis, D.K.R.** and E.C. Boedeker. 1996. Enteric pathogens: Population genetics and pathogenesis of *Escherichia coli* and *Vibrio cholerae* infections. *In*: Gastrointestinal Microbiology. Vol. 2. R.I. Mackie and B.A. White *eds*. Chapman and Hall. Chapter 16, p622-657.

2. Bloom, P.D., **D.K.R. Karaolis**, E.C. Boedeker. 1997. *Escherichia coli* associated diarrhea. *In*: Gastrointestinal Infections. J.Thomas-LaMont *ed*. Marcel Dekker. Chapter 15, p 453-498.

3. **Karaolis, D.K.R.** and J.B. Kaper. 1999. Pathogenicity islands and other mobile virulence elements of *Vibrio cholerae*. *In*: Pathogenicity islands and Other Mobile Virulence Elements. J.B. Kaper and J. Hacker *eds*. ASM Press. Chapter 9, p167-187.

4. **Karaolis, D.K.R.** 2001. Pathogenicity islands. *In*: The Encyclopedia of Genetics. S. Brenner and J.M. Miller *eds*. Academic Press.

JOURNALS (peer reviewed)

1. **Karaolis, D.K.R.**, Lan, R. and Reeves, P.R. 1994. Sequence variation in *Shigella sonnei* (Sonnei), a pathogenic clone of *Escherichia coli*, over four continents and 41 years. *J. Clin. Microbiol.* 32:796-802.

2. **Karaolis, D.K.R.**, Lan, R. and Reeves, P.R. 1994. Molecular evolution of the seventh-pandemic clone of *Vibrio cholerae* and its relationship to other pandemic and epidemic *V. cholerae* isolates. *J. Bacteriol.* 176: 6199-6206.

3. **Karaolis, D.K.R.**, Lan, R. and Reeves, P.R. 1995. The sixth and seventh cholera pandemics are due to independent clones separately derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*. *J. Bacteriol.* 177:3191-3198.

4. **Karaolis, D.K.R.**, Johnson, J.A., Bailey, C.C., Boedeker, E.C., Kaper, J.B., and Reeves, P.R. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. 1998. *PNAS.* 95:3134-3139.

5. Pupo, J., **Karaolis, D.K.R.,** Lan, R. and Reeves, P.R. 1997. Evolutionary relationships among pathogenic and non-pathogenic *Escherichia coli* inferred by MLEE and *mdh* sequence studies. *Infect. Immun.* 65:2685-2692.

6. **Karaolis, D.K.R.**, Somara, S., Maneval, D.R., Johnson, J.A., Kaper, J.B. 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature.* 399:375-379.

7. **Karaolis, D.K.R.**, Lan, R., Kaper, J.B., Reeves, P.R. 2000. A comparison of the *Vibrio cholerae* pathogenicity islands in 6th and 7th pandemic strains. *Infect. Immun.* 69: 1947-1952.

8. Vital Brazil, J.M., Alves, R.M., Rivera, I.N.G., Rodrigues, D.P., **Karaolis, D.K.R.** and Campos, L.C. 2002. Prevalence of virulence-associated genes in clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991-1999. *FEMS Microbiol. Lett.* 215:15-21.

9. Ali, A., Rashid, M. H. and **Karaolis, D.K.R.**. 2002. High frequency rugose exopolysaccharide production in *Vibrio cholerae*. *Appl. Environ. Microbiol.* 68:5773-5778.

10. Zhang, D. Sun, W., Xu, Z. and **Karaolis, D.K.R.** 2003. The VPI-encoded Mop modulates the pathogenesis and reactogenicity of epidemic *Vibrio cholerae in vivo*. *Infect Immun.* 71:510-515.

11. Talledo, M., Rivera, I.N.G., Lipp, E. K., Neale, A., **Karaolis, D.K.R.**, Huq, A., and Colwell, R. R. 2003. Characterization of a *Vibrio cholerae* phage isolated from the coast of Peru. *Environ. Microbiol.* 5:350-354.

12. Zhang, D., Sun, W. and Karaolis, D.K.R.. 2003. Analysis of the *Vibrio* pathogenicity island-encoded Mop protein suggests a pleiotropic role in the virulence of epidemic *Vibrio cholerae*. *FEMS Microbiol. Lett.* 225:311-318.

13. Rashid, M. H., Rajanna, C., Ali, A. and **Karaolis, D. K. R.** 2003. Identification of genes involved in the switch between the smooth and rugose phenotypes of *Vibrio cholerae*. FEMS Microbiol. Letts. 227:113-119.

14. Chythanya, R. Wang, J. Zhang, D. Xu, Z., Ali, A., Hou, Y-M. and **Karaolis, D.K.R.** 2003. The *Vibrio* pathogenicity island of epidemic *Vibrio cholerae* forms precise extrachromosomal circular excision products. *J. Bacteriol.* 185: 6893-6901.

15. Rashid, M.H., Rajanna, C., Zhang, D., Magder, L.S., Ali, A., Dumontet, S., **Karaolis, D. K. R.** 2003. Role of exopolysaccharide, the rugose phenotype and VpsR in the pathogenesis of epidemic *Vibrio cholerae.* FEMS Microbiol. Lett. 230:105-113.

16. Zhang, D.L., Manos, J., Belas, R and **Karaolis, D.K.R.** 2004. Transcriptional analysis and operon structure of the *tagA-orf2-orf3-mop-tagD* region on the *Vibrio* pathogenicity island in epidemic *V. cholerae.* FEMS Microbiol. Lett. 235:199-207.

17. Campos, L.C., Zahner, V., Avelar, K.E.S., Alves, R.M., Pereira, D., Vital Brazil, J.M., Freitas, F.S., Salles, C. A. and **Karaolis, D.K.R.** 2004. Genetic diversity and antibiotic resistance of clinical and environmental *Vibrio cholerae* suggests that many serogroups are reservoirs of resistance. Epidemiol. Infect. 132:985-92.

18. **Karaolis, D.K.R.**, Rashid, M, Rajanna, C., Luo, W., Hyodo, M. Hayakawa, Y. 2005. c-di-GMP (3',5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrobial Agents and Chemotherapy. 49:1029-1038.

19. **Karaolis, D. K. R.**, K. Cheng, M. Lipsky, A. Elnabawi, J. Catalano, M. Hyodo, Y. Hayakawa, and J.-P. Raufman. 2005. 3',5'-cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. Biochem. Biophys. Res. Comm. 329: 40-45.

20. Brouillette, E., Hyodo, M., Hayakawa, Y., **Karaolis, D. K. R.** Malouin, F. 2005. 3',5'-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. Antimicrob. Agents Chemother. 49: 3109-3113.

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EXHIBIT 1

B-249. Role of Cyclic-di-GMP in the Regulation of Brucella Virulence

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Background: *Brucella spp.* are Gram-negative, facultative intracellular bacteria that pose a threat to both animal and human populations. However, little is known about how the bacterium adapts to an intracellular environment and regulates the expression of virulence factors. Recent advances in some gastrointestinal bacteria (*Vibrio spp., E.coli*) have identified a role for the secondary signaling molecule cyclic-di-GMP in adjusting to differing environments and in regulating virulence factor production. After identification of several c-di-GMP regulating genes in the genome of *B. melitensis*, the role of c-di-GMP in pathogenesis was examined. **Methods**: Bioinformatic analysis of the published *B.melitensis* genome found 11 genes that encode domains considered to synthesize or degrade c-di-GMP. Genomic deletion mutants of each of these genes were constructed in *B.melitensis*. These mutants were tested for altered virulence in macrophage and mouse models of infection. Respective proteins were expressed in a *Vibrio parahaemolyticus* model system to evaluate their ability to regulate levels of c-di-GMP. **Results**: While none of the 11 genes hypothesized to regulate cyclic-di-GMP showed any change of infection in macrophages, 3 mutants showed altered virulence in a mouse model. Mutants of 2 genes hypothesized to degrade c-di-GMP were attenuated in mice, while a mutant of a third gene hypothesized to synthesize c-di-GMP was hypervirulent. These proteins were shown to regulate levels of c-di-GMP in the manner hypothesized using a *Vibrio* model system to detect changes in c-di-GMP levels. **Conclusion**: *B.melitensis* deletion mutants of three genes involved in the regulation of c-di-GMP levels showed an altered virulence in mice. Deletion of two genes found to degrade c-di-GMP showed attenuation in mice, while deletion of a gene found to synthesize c-di-GMP showed hypervirulence in a mouse model. This work indicates a role for c-di-GMP in *B.melitensis* virulence in which high levels of c-di-GMP decrease virulence while low levels of c-di-GMP increase virulence.

Acknowledgments/References: This work was supported by NIH/NIAID GLRCE for Biodefense and Emerging Infectious Disease Research Program grant 1U54-AI-057153 and by the Molecular Biosciences Training Grant NIH T32 GM07215-33.

2009 ASM 109th General Meeting Philadelphia, PA

B-249

Role of Cyclic-di-GMP in *Brucella* Virulence

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Abstract

Background: *Brucella* spp. are Gram-negative, facultative intracellular bacteria that pose a threat to both animal and human populations. However, little is known about how the bacterium adapts to an intracellular environment and regulates the expression of virulence factors. Recent advances in some gastrointestinal bacteria (*Vibrio* spp., *E.coli*) have identified a role for the secondary signaling molecule cyclic-di-GMP in adjusting to differing environments and in regulating virulence factor production. After identification of several c-di-GMP regulating genes in the genome of *B. melitensis*, the role of c-di-GMP in pathogenesis was examined. **Methods**: Bioinformatic analysis of the published *B. melitensis* genome found 11 genes that encode domains considered to synthesize or degrade c-di-GMP. Genomic deletion mutants of each of these genes were constructed in *B. melitensis*. These mutants were tested for altered virulence in macrophage and mouse models of infection. Respective proteins were expressed in a *Vibrio parahaemolyticus* model system to evaluate their ability to regulate levels of c-di-GMP. **Results**: While none of the 11 genes hypothesized to regulate cyclic-di-GMP showed any change of infection in macrophages, 3 mutants showed altered virulence in a mouse model. Mutants of 2 genes hypothesized to degrade c-di-GMP were attenuated in mice, while a mutant of a third gene hypothesized to synthesize c-di-GMP was hypervirulent. These proteins were shown to regulate levels of c-di-GMP in the manner hypothesized using a *Vibrio* model system to detect changes in c-di-GMP levels. **Conclusion**: *B. melitensis* deletion mutants of three genes involved in the regulation of c-di-GMP levels showed an altered virulence in mice. Deletion of two genes found to degrade c-di-GMP showed attenuation in mice, while deletion of a gene found to synthesize c-di-GMP showed hypervirulence in a mouse model. This work indicates a role for c-di-GMP in *B. melitensis* virulence in which high levels of c-di-GMP decrease virulence while low levels of c-di-GMP increase virulence.

Brucella

Zoonotic disease of mammals

Infectious cause of brucellosis

Results in spontaneous abortions in animals

Several species are category B select agents and first US-developed biowarfare agent

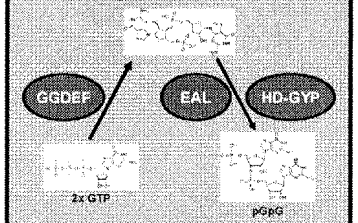
Brucella melitensis

Facultative, intracellular, Gram-negative, bacteria

Prevents fusion of phagosome with lysosome, traffics to ER

Encodes type IV secretory system, putative flagella, non-canonical LPS as virulence factors

Cyclic-di-GMP



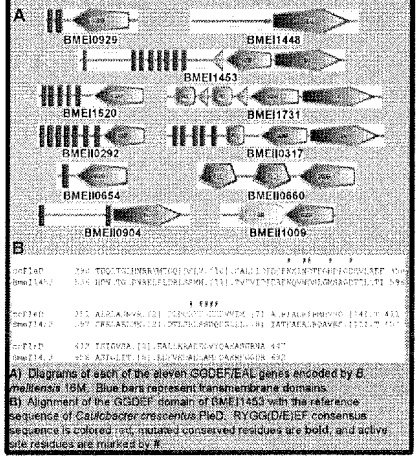
Secondary signalling molecule found in bacteria

Synthesized from two GTP molecules by diguanylate cyclases containing GGDEF domains

Degraded into linear pGpG by phosphodiesterases containing EAL or HD-GYP domains

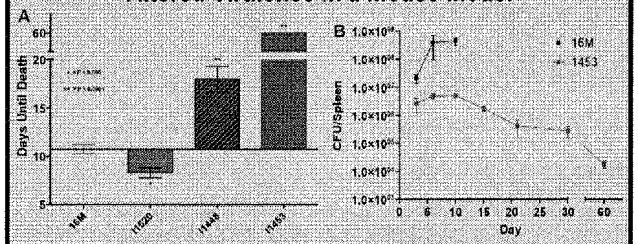
Has been shown to regulate a wide variety of functions in bacteria, including cellulose production, motility, exopolysaccharide synthesis, and virulence

Brucella melitensis 16M Encodes Eleven GGDEF/EAL Genes



A) Diagram of each of the eleven GGDEF/EAL genes encoded by *B. melitensis* 16M. Blue bars represent transmembrane domains.
B) Alignment of the GGDEF domain of BMEI1453 with the reference sequence of *Caulobacter crescentus* PleD. RYGG(D/E)EF consensus sequence is colored red, mutated conserved residues are bold, and active site residues are marked by *.

GGDEF/EAL Deletion Mutants Show Altered Virulence in a Mouse Model



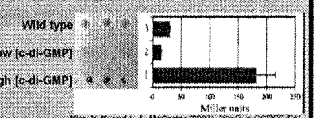
A) Interferon regulatory factor 1 null (IRF-1$^{-/-}$) mice (n=8) were infected with 10^5 CFU of each of the eleven GGDEF/EAL mutant strains and the parent strain. Mice were examined for morbidity and the date of death recorded. Three GGDEF/EAL mutants showed a significant change in virulence in the mouse model.
B) IRF-1$^{-/-}$ mice infected with the parent strain or *BMEI1453* mutant were killed at various timepoints (n=4/timepoint) and the colony forming units/spleen determined. Mice infected with the *BMEI1453* mutant showed decreasing levels of CFUs/spleen, but the mice were unable to eradicate the bacteria by day 60.

A Model System for Cyclic-di-GMP Synthesis/Degradation

Vibrio parahaemolyticus responds to changing c-di-GMP concentrations by regulating biofilm formation, motility, and exopolysaccharide synthesis.

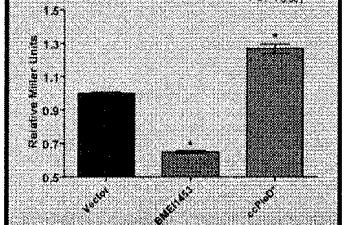
L. L. McCarter has shown that capsular polysaccharide synthesis genes (cps) respond to changes in cyclic-di-GMP concentration.

Integration of *lacZ* into a capsular polysaccharide synthesis gene (*cpsA1::lacZ*) allows for the determination of cyclic-di-GMP concentrations.



Kim, Y. K. and L. L. McCarter (2007). "ScrG, a GGDEF-EAL protein that modulates swarming and stalking in *Vibrio parahaemolyticus*." Journal of Bacteriology **189**(21): 7633-7640.

BMEI1453 Lowers Cyclic-di-GMP Concentrations



Heterologous gene expression in *V. parahaemolyticus* *cpsA1::lacZ* allows for the determination of cyclic-di-GMP concentrations. Miller units show a direct relationship to the concentration of cyclic-di-GMP. Expression of the constitutive mutant of the diguanylate cyclase PleD from *Caulobacter crescentus* results in a higher level of cyclic-di-GMP. Expression of BMEI1453 lowers the concentration of cyclic-di-GMP. Results shown are from two experiments with measurements done in triplicate.

Conclusions

Mutants of *Brucella melitensis* cyclic-di-GMP-regulating proteins show altered virulence in a mouse model.

Deletion mutant of a putative diguanylate cyclase shows an increase in virulence, while deletion mutants of two putative phosphodiesterases show a decrease in virulence.

While wild type *B. melitensis* kills IRF-1$^{-/-}$ mice at approximately 1.5 weeks post infection, the deletion mutant of *BMEI1453* does not kill mice up to 60 days post infection but can be recovered from spleens.

Heterologous expression of BMEI1453 in the *V. parahaemolyticus* model system indicates that BMEI1453 lowers the concentration of cyclic-di-GMP.

Future Directions

Examine other *B. melitensis* proteins/domains in the *V. parahaemolyticus* model system for ability to synthesize or degrade cyclic-di-GMP.

Confirm altered cyclic-di-GMP concentrations in *B. melitensis* GGDEF/EAL deletion mutants.

Identify upstream factors that influence transcription of GGDEF/EAL genes and activation of GGDEF/EAL proteins.

Identify downstream effects of cyclic-di-GMP that affect virulence.

This work was supported by NIH/NIAID GLRCE for Biodefense and Emerging Infectious Disease Research Program grant 1U54-AI-057163 and by the Molecular Biosciences Training Grant NIH T32 GM07215-33.

D-097. c-di-GMP Produced by *E. chaffeensis* Regulates Bacterial Internalization into Host Cells and Intracellular Growth

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Ehrlichia chaffeensis (Ec) is an obligatory intracellular bacterium that causes human monocytic ehrlichiosis. Ec internalizes into host human monocytic leukemia THP-1 cells and proliferates in membrane-bound inclusions in host cells by inducing accumulation of tyrosine-phosphorylated (pTyr) proteins on the inclusions. Ec encodes a single GGDEF domain-containing protein (GCP), PleD response regulator and a cognate sensor kinase PleC. While GCPs are widely distributed in bacteria, functions of c-di-GMP in obligatory intracellular bacteria have not been well characterized due to the lack of useful genetic system. Our hypothesis is c-di-GMP produced by PleD plays roles in Ec intracellular infection. To test the hypothesis, we examined expression of PleC and PleD in synchronous cultured Ec, c-di-GMP production by PleD, and functions of c-di-GMP using a hydrophobic c-di-GMP analog (CDGA), 2'-O-di(tert-butyldimethysilyl)-c-di-GMP. PleC and PleD were synchronously up-regulated at the Ec exponential growth stage and down-regulated prior to Ec extracellular release. Recombinant Ec PleD possessed di-guanylate cyclase activity that produces c-di-GMP when activated by BeF_3^-. When isolated host cell-free Ec were incubated with CDGA, i) some of outer membrane proteins (GP120, OmpA, and VirB6-2) were down-regulated; ii) co-localization with pTyr proteins was abrogated, despite no inhibition of bacterial binding to host cells; and iii) internalization of Ec into host cells was impaired. When CDGA was added to infected cells at the exponential growth stage of Ec, bacterial proliferation was inhibited and the Ec inclusion was malformed. In vitro UV cross-linking with [^{32}P]c-di-GMP revealed c-di-GMP downstream candidate proteins of Ec and the binding of [^{32}P]c-di-GMP to the proteins was competitively inhibited by CDGA. These data suggest that CDGA functions as a c-di-GMP antagonist, and c-di-GMP regulates bacterial outer membrane protein expression, thereby, is involved in signal transduction across host cell membrane for bacterial internalization and intracellular growth.

Acknowledgments/References: This work was supported by the National Institutes of Health grant R01 AI054476. We thank Dr. Stephen Lory, Harvard Medical School (Boston, MA) for the *E. coli* strain over-expressing *Pseudomonas aeruginosa* rWspR, Dr. Matthias Christen in University of Basel (Basel, Switzerland) for detailed methods for c-di-GMP binding assay, Dr. Xue-jie Yu in University of Texas (Galveston, TX) for anti-GP120 antiserum, and Dr. Jere McBride in University of Texas (Galveston, TX) for anti-GP47 antiserum.

DOI: 10.1002/cmdc.200700072

Synthesis of Cyclic Bis(3′-5′)-2′-deoxyguanylic/guanylic Acid (*c*-dGpGp) and Its Biological Activities to Microbes

Erina Mano,[a] Mamoru Hyodo,[a] Yumi Sato,[a] Yuka Ishihara,[b] Michio Ohta,[b] and Yoshihiro Hayakawa*[a]

Cyclic bis(3′-5′)diguanylic acid (*c*-di-GMP) is an important compound with various biological activities, including regulation of cellulose synthesis in the bacterium *Acetobacter xylinum*,[1,2] acceleration of DNA synthesis and retardation of cell division in Molt4 cells,[3] elevation of CD4 receptor expression and cell cycle arrest in Jurkat cells,[4] inhibition of basal and growth factor-stimulated human colon cancer cell proliferation,[5] inhibition of *Staphylococcus aureus* cell–cell interactions and biofilm formation,[6] reduction of the virulence of biofilm-forming *S. aureus* strains in a mouse model of mastitis infection,[7] and activation of the immune response.[8] Furthermore, *c*-di-GMP is considered to play an important role in regulating exopolysaccharide production, biofilm formation, and other phenotypes.[9] These attractive biological properties of *c*-di-GMP prompted us to carry out a systematic study of the bioactivity of *c*-di-GMP-related compounds, including derivatives with modified nucleoside bases, carbohydrates, or internucleotide bonds. This study may lead not only to the discovery of new bioactive compounds, but also to an elucidation of the mechanism by which *c*-di-GMP affects the cell receptors. As a part of this study, we prepared cyclic bis(3′-5′)-2′-deoxyguanylic/guanylic acid (*c*-dGpGp) (**6**) and investigated its effect on the biofilm formation and motility of several bacteria.

c-dGpGp (**6**) was previously synthesized by van Boom and coworkers.[2] However, as they did not describe the experimental details of the synthesis, we could not successfully perform the synthesis according to their method. Therefore, we developed a novel synthetic method for *c*-dGpGp as shown in Scheme 1. The nucleoside phosphoramidite **1**[10] was reacted with allyl alcohol by using imidazolium perchlorate (IMP)[11] as a promoter in the presence of molecular sieves 3A (MS 3A)[12] in acetonitrile (30 min) and the resulting phosphite product was oxidized with a 5 M *tert*-butyl hydroperoxide (TBHP)/decane solution[13] (30 min) to give the nucleoside phosphotriester in 96% yield. This product was treated with a 20% dichloroacetic acid/dichloromethane solution (30 min) to remove the 5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) protector, giving the nucleoside 3′-phosphate **2** in 82% yield. The product **2** was reacted with the phosphoramidite **3** by the aid of IMP in acetonitrile containing MS 3A (30 min), followed by a 5 M TBHP/decane solution (30 min), and then the 5′-*O*-DMTr group of the resulting product was deblocked by a 20% dichloroacetic acid/dichloromethane solution (30 min) to afford the linear (3′-5′)-linked diguanylate **4** in 76% overall yield. Subsequently, the cyanoethyl group on the 3′-terminal phosphotriester moiety of **4** was removed by exposure to diisopropylamine in methanol[14] (2 h), and the resulting product was subjected to intramolecular cyclization using a mixture of 2,4,6-trisipropylbenzenesulfonyl chloride (TPSCl) (5 equiv) and *N*-methylimidazole (5 equiv) (36 h) in THF using a previously reported method[15] to provide fully protected *c*-dGpGp **5** in 91% overall yield. Finally, **5** was reacted with a 1:1 (*v*/*v*) mixture of conc. aqueous ammonia and methanol at 50 °C (12 h) to eliminate the dimethylformamidine (dmf)-protecting group and the allyl-protecting group, followed by $(C_2H_5)_3N{\cdot}3HF$[16] to deblock the *tert*-butyldimethylsilyl (TBDMS)-protecting groups (12 h) to afford the target compound **6** in 40% overall yield.

To consider the biological effects of *c*-dGpGp, we first examined its inhibitory effect (at a high concentration) on the biofilm formation of *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *S. aureus* and compared these levels of inhibition with those by *c*-di-GMP. Experiments were carried out using strains of *P. aeruginosa* PAO1, *S. aureus* MS2507, and *V. parahaemolyticus* ATCC17802. Approximately 10^5 CFUs/mL of *P. aeruginosa* and *S. aureus*, and 10^7 CFU mL^{-1} of *V. parahaemolyticus* were incubated in L broth for *P. aeruginosa* and *S. aureus* and heart infusion broth for *V. parahaemolyticus* supplemented with 200 μM of *c*-dGpGp and 200 μM of *c*-di-GMP, respectively, in the wells of a polystyrene microtiter plate without shaking at 30 °C for 24 h. Subsequently, dishes were washed with saline twice and stained with crystal violet as described previously.[6,17] As control, aliquots of the microbes were incubated under the same conditions but without the nucleic acid treatment. Measurements were performed in triplicate. Experiments were repeated at least twice.

The effect of *c*-dGpGp and *c*-di-GMP on bacterial biofilm formation is shown in Figure 1. Both cyclic dinucleotides suppressed the biofilm formation of the three different kinds of bacteria, although the activity of *c*-dGpGp was less than that of *c*-di-GMP. The difference in the inhibitory activity should not be due to the growth suppression of bacteria, as the number of viable bacteria (colony forming unit, CFU) after the treatment with *c*-dGpGp and *c*-di-GMP were not different from those of control experiments (data not shown).

According to a previous study[18] using a microbe with flagella, motility and the ability to form biofilm are closely related. Therefore, we subsequently investigated the effect of *c*-dGpGp and *c*-di-GMP on the motility of the three bacteria, which belong to different groups in the bacterial taxonomy. The examinations were performed using *P. aeruginosa* PAO1, *Salmonella enterica* serovar Typhimurium LT2, and *V. parahaemolyticus* ATCC17802 according to the methods described in a previous study.[19,20] The motility of *P. aeruginosa* and *S. typhimurium* were measured in the presence of 200 μM of *c*-di-GMP and *c*-

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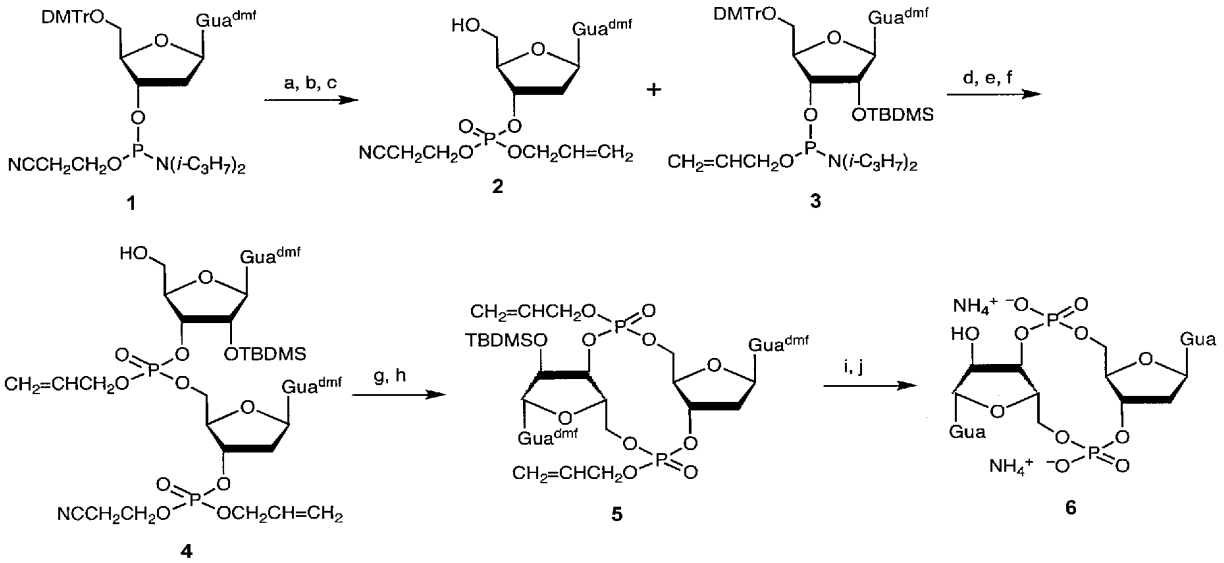

Scheme 1. a) allyl alcohol, IMP, MS 3A, CH_3CN, 25 °C, 30 min; b) 5–6 M TBHP/decane solution, 25 °C, 30 min; c) 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 30 min; d) IMP, MS 3 A, CH_3CN, 25 °C, 30 min; e) 5–6 M TBHP/decane solution, 25 °C, 30 min; f) 20% $Cl_2CHCOOH/CH_2Cl_2$ solution, 25 °C, 30 min; g) $(iPr)_2NH$-CH_3OH (1:1 *v/v*), 25 °C, 1 h; h) TPSCl, *N*-methylimidazole; 25 °C, 36 h; i) conc. aq. NH_3-CH_3OH (1:1 *v/v*), 50 °C, 12 h; j) $(C_2H_5)_3N{\cdot}3HF$, 25 °C, 12 h.

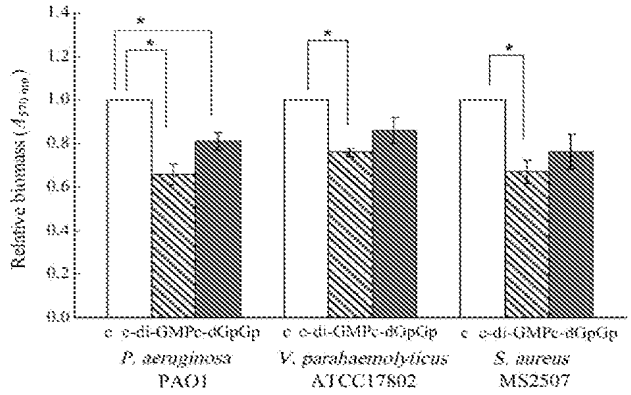

Figure 1. Effect of cyclic-dinucleotides on biofilm formation. Biofilm was stained with crystal violet and solubilized with dimethyl sulfoxide to measure at OD_{570}. Longitudinal axis expresses relative biomass to control. * $P < 0.05$.

Table 1. Effect of c-di-GMP and c-dGpGp on motility of some bacteria.

Strain	Change of motility[a]	
	treatment with *c*-di-GMP	treatment with *c*-dGpGp
P. aeruginosa PA01	8% promotion[b]	17% repression[b]
S. typhimurium LT2	37% repression[b]	29% repression[b]
V. parahaemolyticus ATCC17802	60% promotion[c]	10% repression[c]

[a] Comparison with the sample untreated with the cyclic dinucleotide. [b] Treated with 200 mM c-di-GMP and c-dGpGp. [c] Treated with 100 mM c-di-GMP and c-dGpGp.

dGpGp, and that of *V. parahaemolyticus* was measured in the presence of 100 μM of *c*-di-GMP and *c*-dGpGp. These bacteria were incubated on plates of 0.3% agar L broth containing each concentration of c-di-GMP and c-dGpGp at 30 °C for 20 h and the diameter of the swimming was measured as the index of motility. As control, aliquots of the three microbes were incubated under the same conditions but without the cyclic dinucleotides. The resulting motility patterns are shown in Figure 2. Further, Table 1 summarizes the mobility of the treated bacteria relative to that of the untreated bacteria. Thus, these examinations revealed that *c*-di-GMP promoted the motility of *P. aeruginosa* and *V. parahaemolyticus*, but repressed the motility of *S. typhimurium*; on the other hand, *c*-dGpGp weakly repressed the motility of all of the bacteria.

We next examined why *c*-dGpGp and *c*-di-GMP should exhibit different biological activities despite their similar structures. Although a number of explanations are conceivable, the most feasible one is that *c*-dGpGp and *c*-di-GMP have different three-dimensional conformations and thus different binding affinity to receptors (target molecules). Thus, the most stable conformations of *c*-dGpGp and *c*-di-GMP were analyzed by the B3LYP/6-31 + G(d,p)level MO calculation method using Spartan 04. Figure 3 exhibits the results. Comparison of the resulting structures indicated that the conformation of one of the two guanine groups was quite different between *c*-dGpGp and *c*-di-GMP. As the obtained conformations were not those in water, we propose—but cannot definitively state—that the conformational difference in *c*-dGpGp and *c*-di-GMP may be one of the factors causing their different biological properties.

As a part of our ongoing investigation of the biological activity of *c*-di-GMP and its analogues, we prepared *c*-dGpGp (**6**) and investigated the biological activities of this compound in comparison with those of *c*-di-GMP. The investigation disclosed that both *c*-dGpGp and *c*-di-GMP inhibit the biofilm formation

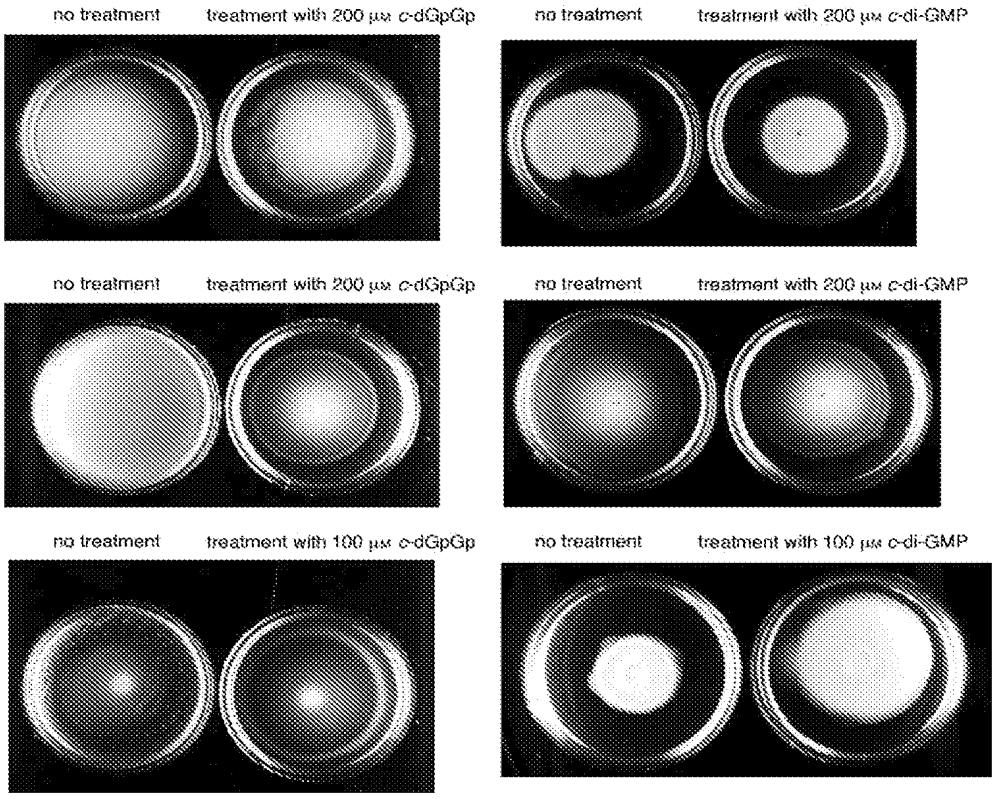

Figure 2. Motility of *P. aeruginosa*, *S. typhimurium*, and *V. parahaemolyticus* treated with and without c-dGpGp and c-di-GMP. Upper panel: *P. aeruginosa*; middle panel: *S. typhimurium*; lower panel: *V. parahaemolyticus*.

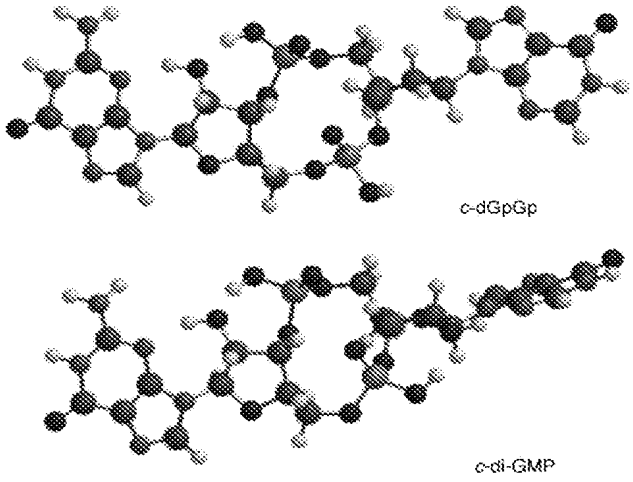

Figure 3. The most stable comformations of c-dGpGp and c-di-GMP.

of *P. aeruginosa*, *V. parahaemolyticus*, and *S. aureus*, and the inhibitory effect of *c*-di-GMP is higher, though not significant, than that of *c*-dGpGp. Further, it was revealed that *c*-dGpGp slightly represses the motility of *P. aeruginosa*, *V. parahaemolyticus*, and *S. typhimurium*. The observed activities for *P. aeruginosa* and *V. parahaemolyticus* were different from those of *c*-di-GMP. That is, *c*-di-GMP promoted the motility of *P. aeruginosa* and *V. parahaemolyticus*. These findings suggest that certain analogues of *c*-di-GMP, much like those of *c*-di-GMP, may have good potential as antibacterial agents, and thus further investigations on the biological properties of various *c*-di-GMP analogues are necessary. Among the discoveries in the present work, the fact that both the motility and biofilm-formation ability were depressed in *V. parahaemolyticus* treated with *c*-dGpGp may be the most attractive and important because previous examinations have suggested that enzymes that produce biofilm and those that enhance the motility of microbes do not work simultaneously. Thus, when biofilm formation occurs, the microbe stops its movement. Conversely, when the microbe moves, biofilm formation is stopped. Therefore, microbe motility should be promoted in microbes, in which biofilm formation is inhibited. However, the present experiments were not designed to examine this hypothesis. This result has motivated us to carry out extensive and systematic investigations for elucidating the true relationship between biofilm-forming ability and motility of microbes with flagella.

Acknowledgements

This study was partly supported by Grants-in-Aid for Scientific Research (No. 16011223) and for the 21st Century COE Program (Establishment of COE of Materials Science: Elucidation and Creation of Molecular Functions) from the Ministry of Education, Culture, Science, Sports and Technology of Japan. This work was also supported by CREST of JST (Japan Science and Technology).

Keywords: biological activity · *c*-di-GMP · nucleotides · structure–activity relationships

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Received: March 28, 2007
Revised: July 24, 2007

